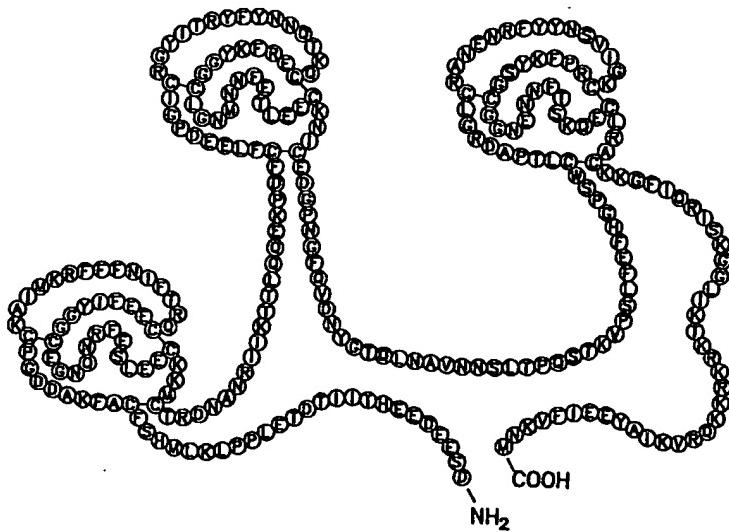




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(54) Title: ANTICOAGULANT PROTEIN



(57) Abstract

Novel EPI analogues are provided wherein one or more of the amino acid residues of native EPI have been deleted. A preferred group of the novel EPI analogues have no or a low heparin binding capacity. The novel EPI analogues can be used for the treatment of patients having coagulation disorders or cancer.

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ANTICOAGULANT PROTEINField of the invention

The present invention relates to a new protein with EPI activity, a method of producing such protein and a therapeutic preparation containing such protein.

Background of the invention

Blood coagulation is a complex process involving many activating and inactivating coagulation factors. Anticoagulant proteins are known to be important for regulation of the coagulation process (see B. Lämmle and J. Griffin (Clinics in Haematology 14 (1985), 281-342) and anticoagulants are thus important in the treatment of a variety of diseases, eg thrombosis, myocardial infarction, disseminated intravascular coagulation etc.

Thus heparin is used clinically to increase the activity of antithrombin III and heparin cofactor II. Antithrombin III is used for the inhibition of factor Xa and thrombin. Hirudin is used for the inhibition of thrombin and protein C may be used for the inhibition of factor V and factor VIII.

Anticoagulant proteins may also be used in the treatment of cancer. Thus, antistatin has been shown to have anti-metastatic properties (J.H. Han et al., Gene 75 (1989), 47-57). Also heparin and warfarin have been shown to possess antimetastatic properties (G.J. Gasic et al., Int. Rev. Exp. Pathol. 29 (1985), 173-209).

Coagulation can be initiated through the extrinsic pathway by the exposure of tissue factor (TF) to the circulating blood (Y. Nemerson, Blood 71 (1988), 1-8). Tissue factor is a protein cofactor for factor VII/VIIa and binding of tissue factor enhances the enzymatic activity of factor VIIa (FVIIa) towards

its substrates factor IX and factor X. Placenta anticoagulant protein is able to inhibit tissue factor activity, probably by interfering with TF/FVIIa-phospholipid interaction (S. Kondo et al., Thromb. Res. 48 (1987), 449-459).

5 Recently a new anticoagulant protein, the extrinsic pathway inhibitor (EPI) has been isolated (Broze et al., Proc. Natl. Acad. Sci. 84 (1987), 1886-1890).

On a molar basis EPI has been shown to be a potent inhibitor of TF/FVIIa induced coagulation (R.A. Gramzinski et al., Blood 73 10 (1989), 983-989). EPI binds and inhibits factor Xa (FXa) and the complex between EPI and FXa inhibits TF/FVIIa (Rapaport, Blood 73 (1989), 359-365). EPI is especially interesting as an anticoagulant/antimetastatic agent as many tumor cells express TF activity (T. Sakai et al., J. Biol. Chem. 264 (1989), 9980-15 9988) and because EPI shows anti-Xa activity like antistatin.

EPI has been recovered by Broze et al. (supra) from HepG2 hepatoma cells (Broze EP A 300988) and the gene for the protein has been cloned (Broze EP A 318451). A schematic diagram over the secondary structure of EPI is shown in Fig. 1 and the amino acid sequence of EPI is shown in Fig. 2 where the N-terminal amino acid Asp is given the number 1. The protein consists of 276 amino acid residues and has in addition to three inhibitor domains of the Kunitz type three potential glycosylation sites at position Asn117, Asn167 and Asn229. The molecular weight 20 shows that some of these sites are glycosylated. Furthermore, it has been shown that the second Kunitz domain binds FXa while the first Kunitz domain binds FVIIa/TF (Girard et al., Nature 338 (1989), 518-520). EPI has also been isolated from HeLa cells (PCT/DK90/00016) and it was shown that HeLa EPI binds 25 heparin.

Heparin binding is an important factor for the pharmacokinetics of substances for injection. It has been shown that platelet factor 4 (M. Prosdomi et al., Thromb. Res. 39 (1985), 541-

547) and aprotinin with one Kunitz domain (H. Fritz et al., Drug Res. 33, 479-94) has a short half life probably due to the heparin binding properties. Lowering of the heparin binding capacity of an anticoagulant would therefore seem to be 5 advantagous. Furthermore, it might be advantagous to use a smaller molecule than EPI for the medical treatment.

It has according to the present invention surprisingly been found that certain EPI analogues retain the EPI activity as well as anti Xa activity although parts of the molecule has 10 been deleted. Furthermore, these analogues show a much lower affinity for heparin than full length EPI, making them more useful as therapeutic agents than the native molecule. The EPI analogues will furthermore have a longer half life as compared with native EPI which will further reduce the amount of active 15 ingredients for the medical treatment.

Summary of the invention

In its first aspect the present invention is related to novel EPI analogues wherein one or more of the amino acid residues of native EPI have been deleted.

20 In its second aspect the present invention is related to a new group of EPI analogues having EPI activity but with no or low heparin binding capacity under physiological conditions (pH, ionic strength).

In the present context the term "low heparin binding capacity" 25 is intended to mean a binding capacity of about 50%, more preferably of about 25% and most preferably less than about 10% of that of native EPI at physiological pH and ionic strength.

The preferred group of the novel EPI analogues can be characterized as being devoid of the heparin binding domain of native 30 EPI or having a non-functional heparin binding domain by having deleted one or more of the amino acid residues in said

domain resulting in loss or a substantial lowering of the heparin binding capacity. The same effect may also be obtained by substituting one or more of the amino acid residues in the heparin binding domain with another amino acid residue.

5 Detailed description of the invention

To retain the EPI activity the analogues according to the present invention should at least contain the N-terminal sequence including the first and second Kunitz domain. Thus, the EPI analogues according to the present invention should at least contain the amino acid sequence from amino acid number 25 to amino acid number 148 of native EPI (see fig. 1 and 2).

It has been shown by the inventors hereof that the heparin binding capacity is lost when the sequence from amino acid residue number 162 to amino acid residue number 275 is deleted from the EPI molecule. It is therefore concluded that the heparin binding domain is situated in this part of the EPI molecule. It is assumed that the heparin binding domain comprises at least a region from Arg246 to Lys265 near the C-terminal end of the EPI molecule being rich in positively charged amino acid residues.

Preferred EPI analogues according to the present invention are such in which one or more amino acid residues have been deleted in the native EPI molecule from amino acid residue Glu148 to the C-terminal Met276.

More specifically, one or more amino acid residues in the sequence from Arg246 to Lys275 have been deleted.

Examples of EPI analogues according to the present invention are:

(Asp1 - Thr255)-EPI
(Asp1 - Ile253)-EPI
(Asp1 - Lys249)-EPI
(Asp1 - Ser248)-EPI
5 (Asp1 - Lys240)-EPI
(Asp1 - Glu234)-EPI
(Asp1 - Trp188)-EPI
(Asp1 - Asn164)-EPI
(Asp1 - Thr161)-EPI
10 (Asp1 - Asp149)-EPI
(Asp1 - Glu148)-EPI

Ser-(Asp1 - Thr255)-EPI
Ser-(Asp1 - Ile253)-EPI
Ser-(Asp1 - Lys249)-EPI
15 Ser-(Asp1 - Ser248)-EPI
Ser-(Asp1 - Lys240)-EPI
Ser-(Asp1 - Glu234)-EPI
Ser-(Asp1 - Trp188)-EPI
Ser-(Asp1 - Asn164)-EPI
20 Ser-(Asp1 - Thr161)-EPI
Ser-(Asp1 - Asp149)-EPI
Ser-(Asp1 - Glu148)-EPI

(Glu15 - Thr255)-EPI
(Glu15 - Ile253)-EPI
25 (Glu15 - Lys249)-EPI
(Glu15 - Ser248)-EPI
(Glu15 - Lys240)-EPI
(Glu15 - Glu234)-EPI
(Glu15 - Trp188)-EPI
30 (Glu15 - Asn164)-EPI
(Glu15 - Thr161)-EPI
(Glu15 - Asp149)-EPI
(Glu15 - Glu148)-EPI

Ser-(Glu15 - Thr255)-EPI
Ser-(Glu15 - Ile253)-EPI
Ser-(Glu15 - Lys249)-EPI
Ser-(Glu15 - Ser248)-EPI
5 Ser-(Glu15 - Lys240)-EPI
Ser-(Glu15 - Glu234)-EPI
Ser-(Glu15 - Trp188)-EPI
Ser-(Glu15 - Asn164)-EPI
10 Ser-(Glu15 - Thr161)-EPI
Ser-(Glu15 - Asp149)-EPI
Ser-(Glu15 - Glu148)-EPI

(Ser24 - Thr255)-EPI
(Ser24 - Ile253)-EPI
(Ser24 - Lys249)-EPI
15 (Ser24 - Ser248)-EPI
(Ser24 - Lys240)-EPI
(Ser24 - Glu234)-EPI
(Ser24 - Trp188)-EPI
(Ser24 - Asn164)-EPI
20 (Ser24 - Thr161)-EPI
(Ser24 - Asp149)-EPI
(Ser24 - Glu148)-EPI

(Asp1 - Thr255)-(Ile266 - Met276)-EPI
(Asp1 - Ile253)-(Ile266 - Met276)-EPI
25 (Asp1 - Ser248)-(Ile266 - Met276)-EPI
(Asp1 - Gln245)-(Ile266 - Met276)-EPI
(Asp1 - Thr255)-(Val264 - Met276)-EPI
(Asp1 - Ile253)-(Val264 - Met276)-EPI
(Asp1 - Ser248)-(Val264 - Met276)-EPI
30 (Asp1 - Glu245)-(Val264 - Met276)-EPI
(Asp1 - Thr255)-(Glu262 - Met276)-EPI
(Asp1 - Ile253)-(Glu262 - Met276)-EPI
(Asp1 - Ser248)-(Glu262 - Met276)-EPI
(Asp1 - Glu245)-(Glu262 - Met276)-EPI

Ser-(Asp1 - Thr255)-(Ile266 - Met276)-EPI
Ser-(Asp1 - Ile253)-(Ile266 - Met276)-EPI
Ser-(Asp1 - Ser248)-(Ile266 - Met276)-EPI
Ser-(Asp1 - Gln245)-(Ile266 - Met276)-EPI
5 Ser-(Asp1 - Thr255)-(Val264 - Met276)-EPI
Ser-(Asp1 - Ile253)-(Val264 - Met276)-EPI
Ser-(Asp1 - Ser248)-(Val264 - Met276)-EPI
Ser-(Asp1 - Glu245)-(Val264 - Met276)-EPI
Ser-(Asp1 - Thr255)-(Glu262 - Met276)-EPI
10 Ser-(Asp1 - Ile253)-(Glu262 - Met276)-EPI
Ser-(Asp1 - Ser248)-(Glu262 - Met276)-EPI
Ser-(Asp1 - Glu245)-(Glu262 - Met276)-EPI

In addition to the described deletions in the EPI molecule, certain amino acid residues of native EPI may also be replaced 15 by another naturally occurring amino acid residue. The EPI analogues may also advantageously contain a Ser residue as the N-terminal residue. This is necessary if a signal sequence is used requiring an N-terminal Ser in the mature protein as a recognition site for cleavage. Thus, the N-terminal in the EPI 20 molecule may be replaced by a Ser or an additional Ser may be inserted adjacent to the original N-terminal residue. Also the potential glycosylation sites at Asn167 and Asn238 may be substituted by another amino acid residue to avoid glycosylation.

25 It has not previously been shown that glycosylation sites are dispensable for EPI activity. Neither has it been shown that large fragments of the EPI protein are dispensable for EPI activity, i.e. for FXa dependent inhibition of TF/FVIIa. It has previously been shown that a single amino acid in EPI can be 30 replaced by another amino acid residue (Arg199 -> Leu199), (Girard et al., *Nature* 338 (1989), 518-520) without affecting the activity. However, such a change does not significantly change the structure of the protein. In contrast hereto the deletions according to the present invention give rise to new

molecules with other properties than the native molecule, as illustrated by the changed affinity for heparin.

The present EPI-analogues may be produced by well-known recombinant DNA technology.

5 The gene for the native EPI has been cloned and sequenced (Wun et al., J. Biol. Chem. 263 (1988), 6001-6004). DNA-sequences encoding the desired EPI-analogue may be constructed by altering EPI cDNA by site-directed mutagenesis using synthetic oligonucleotides encoding the desired amino acid sequence for 10 homologous recombination in accordance with well-known procedures.

The DNA sequence encoding the EPI analogue of the invention may also be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by S.L. Beaucage and 15 M.H. Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or the method described by Matthes et al., EMBO Journal 3, 1984, pp. 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable 20 vectors.

In a further aspect, the present invention relates to a recombinant expression vector which comprises a DNA sequence encoding the EPI analogue of the invention. The expression vector may be any vector which may conveniently be subjected to 25 recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, 30 e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence encoding the EPI analogue of the invention should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and 5 may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA encoding the EPI analogue of the invention in mammalian cells are the SV 40 promoter (Subramani et al., Mol.Cell Biol. 1, 1981, pp. 854-10 864), the MT-1 (metallothionein gene) promoter (Palmiter et al., Science 222, 1983, pp. 809-814), the adenovirus 2 major late promoter or the CMV (cytomegalovirus IE1) promoter (Henninghausen et al., EMBO J. 5 (1986), 1367-71). Suitable promoters for use in yeast host cells include promoters from 15 yeast glycolytic genes (Hitzeman et al., J.Biol.Chem. 255, 1980, pp. 12073-12080; Alber and Kawasaki, J.Mol.Appl.Gen. 1, 1982, pp. 419-434) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals (Hollaender et al., eds.), Plenum Press, New York, 1982), or 20 the PTI1 (US 4,599,311) or ADH2-4C (Russell et al., Nature 304, 1983, pp. 652-654) promoters. Suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (McKnight et al., The EMBO J. 4, 1985, pp. 2093-2099) or the tipA promoter.

25 To ensure secretion a suitable signal sequence is inserted at the 5' of the DNA sequence encoding the EPI analogue. A suitable signal sequence is the t-PA signal sequence (Friezner et al., J.Biol.Chem. 261 (1986), 6972-85).

The DNA sequence encoding the EPI analogue of the invention 30 should also be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., op. cit.) r (for fungal hosts) the PTI1 (Alber and Kawasaki, op. cit.) r ADH3 (McKnight et al., op. cit.) promoters. The vector may further comprise elements such as polyadenylation

signals (e.g. from SV 40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the SV 40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs).

5 The recombinant expression vector of the invention may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV 40 origin of replication. The vector may also comprise a selectable marker, 10 e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or one which confers resistance to a drug, e.g. neomycin, hygromycin or methotrexate.

The procedures used to ligate the DNA sequences coding for the 15 EPI analogue of the invention, the promoter and the terminator, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., op. cit.).

20 In a further aspect, the present invention relates to a cell which contains the recombinant expression vector described above. The host cell may be any cell which is capable of producing the EPI analogue and is preferably a eukaryotic cell, in particular a mammalian cell. Examples of suitable mammalian 25 cell lines are the COS (ATCC CRL 1650 and 1651), BHK (ATCC CRL 1632, ATCC CCL 10) or CHO (ATCC CCL 61) cell lines. Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, J.Mol.Biol. 159, 1982, pp. 601-621; Southern and Berg, 30 J.Mol.Appl.Genet. 1, 1982, pp. 327-341; Loyter et al., Proc.Natl.Acad.Sci. USA 79, 1982, pp. 422-426; Wigler et al., Cell 14, 1978, p. 725; Corsaro and Parson, Somatic Cell Genetics 7, 1981, p. 603, Graham and van der Eb, Virology 52, 1973, p. 456; and Numan et al., EMBO J. 1, 1982, pp. 841-845.

Alternatively, fungal cells (including yeast cells) may be used as host cells of the invention. Examples of suitable yeast cells include cells of Saccharomyces spp. or Schizosaccharomyces spp., in particular strains of 5 Saccharomyces cerevisiae. Examples of other fungal cells are cells of filamentous fungi, e.g. Aspergillus spp. or Neurospora spp., in particular strains of Aspergillus oryzae or Aspergillus niger. The use of Aspergillus spp. for the expression of proteins is described in, e.g., EP 272,277.

10 In a still further aspect the present invention relates to a process for producing an EPI analogue according to the invention, which comprises culturing a cell as described above in a suitable nutrient medium under conditions which are conducive to the expression of the EPI analogue, and 15 recovering the polypeptide from the culture. The medium used to culture the cells may be any conventional medium suitable for growing mammalian cells, such as a serum-containing or serum-free medium containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared 20 according to published recipes (e.g. in catalogues of the American Type Culture Collection).

The EPI analogue will preferably be secreted to the growth medium and may be recovered from the medium by conventional procedures including separating the host cells from the medium 25 by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g ammonium sulphate, followed by purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, affinity chromatography, or the 30 like.

The novel EPI analogues may be used for the treatment of patients having coagulation disorders or cancer.

Accordingly the invention is also related to a pharmaceutical preparation for the treatment of patients having coagulation disorders or cancer containing an EPI analogue in a suitable amount together with suitable adjuvants and additions.

5 The pharmaceutical preparations may be in a buffered aqueous solution with appropriate stabilizers and preservatives. The solution may be heat treated and may be contained in ampoules or in carpoules for injection pens. Alternatively the stabilized solution may be freeze dried and contained in 10 ampoules or in two chamber injection systems with freeze dried substanse in one chamber and solvent in the other chamber.

Brief description of the Drawings

The present invention is further described with reference to the drawings in which

15 Fig. 1 shows the amino acid sequence and the two dimensional structure of EPI,

Fig. 2 shows the amino acid sequence of native EPI,

20 Fig. 3 shows plasmid pJR77 containing the t-PA signal, the human cytomegalovirus IE1 gene promoter and the human growth hormone gene polyadenylation signal,

Fig. 4 shows pCMVEPIhGH containing the EPI gene,

Fig. 5 shows plasmid pEP1b1 containing a DNA sequence encoding Ser-(Thr88 - Thr161)-EPI,

25 Fig. 6 shows plasmid PEP1ab containing a DNA sequence encoding Ser-(Glu15 - Thr161)-EPI,

Fig. 7 shows th DNA s qu nc for Ser-(Thr88 - Thr161)-EPI preceded by the t-PA signal sequence,

Fig. 8 shows the DNA-sequence for the Ser-(Glu¹⁵ - Thr¹⁶¹)-EPI preceded by the t-PA signal sequence,

Fig. 9 shows plasmid PEPIab2 containing a DNA sequence encoding (Asp¹ - Thr¹⁶¹)-EPI, and

5 Fig. 10 shows the DNA sequence for (Asp¹ - Thr¹⁶¹)-EPI preceded by the EPI signal.

The invention is further described in the following examples which are not in any ways intended to limit the scope or spirit of the invention as claimed.

10 Experimental Part

Assay for EPI activity: EPI was measured in a chromogenic microplate assay, modified after the method of Sandset et al., (Thromb. Res. 47 (1989), 389-400). Heat treated plasma pool was used as a standard. This standard is set to contain 1 U/ml 15 of EPI activity. Standards and samples were diluted in buffer A (0.05 M tris / 0.1 M NaCl / 0.1 M Na-citrate / 0.02% NaN₃ / pH 8.0) containing 2 µg/ml polybrene and 0.2% bovine serum albumin. FVIIa/TF/FX/CaCl₂ combination reagent was prepared in buffer A and contained 1.6 ng/ml FVIIa (Novo-Nordisk a/s), 20 human tissue factor diluted 60 fold (Hjort, Scand. J. Clin. Lab. Invest. 9 (1957), 50 ng/ml FX (Sigma) and 18 mM CaCl₂. The assay was performed in microplate strips at 37°C. 50 µl of samples and standards were pipetted into the strips and 100 µl combination reagent was added to each well. After 10 minutes 25 incubation, 25 µl of FX (3.2 µg/ml) was added to each well and after another 10 minutes 25 µl of chromogenic substrate for FXa (S2222) was added 10 minutes after the addition of substrate. The reaction was stopped by addition of 50 µl 1.0 M citric acid pH 3.0. The microplate was read at 405 nm.

Assay for anti Xa activity: HeLa EPI purified on heparin sepharose (PCT/DK90/00016) was used as a standard. This standard was assigned an amount of Xa inhibition units corresponding to the amount of EPI units measured in the EPI assay. Samples and 5 standards were diluted in 50 mM tris/0.2% bovine serum albumin pH 7.3. 100 µl of diluted samples and standards were incubated 30 minutes at 37°C with 100 µl FXa (Stago, 14 ng/ml). 25 µl of S2222 (2 mg/ml) was added after another 2 hours at 37°C. The assay was stopped and read like the EPI assay.

10 Synthetic oligonucleotides were synthesized by the phosphoramidite method using an Applied Biosystem DNA synthesizer.

M13 sequencing primers and gamma-[³²P]-ATP (5000 Ci/mmol, 10 mCi/ml) for labelling of primers were obtained from Amersham.

15 Restriction endonucleases and T4 DNA-ligase were obtained from New England Biolabs. Modified T7 DNA-polymerase (Sequenase) was obtained from United States Biochemicals. pBS+ (Stratagene) was used as cloning vector for synthetic DNA fragments.

A mammalian expression vector denoted pJR77 (fig. 3) containing the human cytomegalovirus IE1 gene promoter and the human 20 growth hormone gene polyadenylation signal was used for expression of various EPI related proteins in COS-7 cells.

XL-1 Blue (Stratagene) a derivative of E. coli K12 was used as bacterial recipient for plasmid transformations and as host for propagation and preparation of plasmid DNA.

25 Green monkey kidney cell-line COS-7 (ATCC # CRL 1651) was grown in Dulbecco's modified eagle medium (DMEM) (Gibco 041-1965) + 50 µg/ml gentamycin + 110 µg/ml pyruvate + 10% fetal calf serum (FCS) or DMEM + 50 µg/ml gentamycin + 110 µg/ml pyruvate + 1% ITS* (insulin, transferrin, serum albumin).

Restriction endonucleases and other enzymes were used in accordance with the manufacturers recommendations. Standard recombinant DNA-techniques were carried out as described (Maniatis et al., Molecular cloning. Cold Spring Harbor Laboratory, 1982).

DNA sequences were determined by the dideoxy chain termination method (Sanger et al., Proc. Natl. Acad. Sci. 74 (1977), 5463-67) using double stranded plasmid DNA as template and ³²P-labelled primers and Sequenase.

10 Plasmid DNA was introduced into COS-7 cells by calcium phosphate coprecipitation (Graham & van der Eb, Virology 52 (1973), 456-457).

7x10⁵ cells were seeded in 20 cm² dishes in 5 ml DMEM + FCS. The following day each dish was added 20 µg plasmid DNA in 0.5 15 precipitate and 50 µl 10 mM chloroquine diphosphate. Cells were incubated overnight with precipitate. The following day fresh medium DMEM + ITS⁺ was added. After two days of incubation media were harvested and assayed for EPI-activity and anti Xa-activity.

20 Example 1

Preparation of DNA-fragments encoding EPI and EPI analogues and transient expression in COS-7 cells.

The EPI cDNA sequence is described by Wun et al. (J. Biol. Chem. 263 (1988), 6001-6004). Based on the known sequence 25 synthetic DNA-sequences encoding EPI and EPI analogues were constructed by silent mutations introducing restriction endonuclease recognition sites at suitable locations.

Expression cassettes encoding EPI fragments using the tPA signal sequence (fig. 7 and 8) and an expression cassette 30 coding the entire EPI prot in were assembled by sequentially insertion into the cloning vect r pBS+ of annealed complemen-

tary synthetic oligonucleotides. Following assembly of DNA fragment sequences were verified by DNA sequencing as described.

Finally expression cassettes were transferred to expression vector pJRK77 giving rise to the following expression plasmids:

pEPIb1 encoding the tPA signal followed by EPI analogue Ser-(Thr88 - Thr161)-EPI.

pEPIab encoding the tPA signal followed by EPI analogue Ser-(Glu15 - Thr161)-EPI.

10 pCMVEPIhGH encoding the entire EPI protein.

COS-7 cells were transfected as indicated in Table 1. After 2 days of incubation with DMEM + ITS⁺ media were harvested. Table 1 shows assay results for EPI-activity and anti-Xa activity.

Table 1.

15

	Plasmid DNA	EPI [U/ml]	anti-Xa [U/ml]
20	pBS+	0.19	0.19
	pCMVEPIhGH	8.3	7.6
	pEPIb1	0.28	0.15
	pEPIab	4.5	5.6

The results in table 1 show that the EPI-fragment Ser-(Thr88 - Thr161)-EPI containing only the second Kunitz domain has no activity whereas the EPI-fragment Ser-(Glu15 - Thr161)-EPI containing the first and second Kunitz domain has retained the EPI activity.

Example 2

COS transfections with EPI plasmids were performed as described in example 1 and media were harvested after 48 hours. 5 columns were packed, each with 300 ul of heparin sepharose. 5 The columns were equilibrated with 20 mM tris/10% glycerol, pH 7.5. (buffer B). 1.3 ml culture medium was applied to each column. Then the columns were washed with 1.5 ml buffer B and each column was eluted with steps of 1.5 ml buffer B with increasing amounts of NaCl. For comparison is given data from 10 another experiment where EPI from HeLa and HepG2 cells were fractionated on heparin-sepharose. In this experiment other NaCl concentrations were used for elution. Table 2 shows that Ser-(Glu₁₅ - Thr₁₆₁)-EPI does not bind to heparin at physiological pH and ionic strength.

Table 2. Heparin binding of EPI and EPI-like proteins

5	Culture medium	U EPI in medium	flow through	% of EPI activity				
				B wash	M 0.25	M 0.5	M 0.75	M 2.0
10	untransfected COS	0.25	<4	<4	<4	17	54	33
15	EPI-transfected COS	7.10	<4	<4	<4	6	48	<4
20	Ser-(Glu15-Thr161)-EPI transfected COS	8.34	76	24	<3	<3	<3	<3
25	untransfected HeLa	1.17	NM	NM	ND	64	ND	15
	untransfected HepG2	1.95	NM	NM	ND	12	ND	74

NM: not measured, ND: not done

Example 3Preparation of (Asp1 - Thr161)-EPI

Construction of expression plasmids, transformation and expression in COS-7 cell was performed using materials and methods as described in Example 1.

Sequences between SalI and BamHI of expression plasmid pEPIab (Fig. 6) encoding the t-PA signal and Ser-(Glu15 - Thr161)-EPI was replaced by a synthetic DNA-sequence encoding the authentic EPI signal and (Asp1 - Thr161)-EPI.

The resulting plasmid pEPIab2 is shown in Fig. 9 and the expression cassette of pEPIab2 is shown in Fig 10.

Culture medium from pEPIab2 transfected COS cells were applied to Heparin-Sepharose as described in Example 2. 3.8 ml culture medium containing 31.2 U/ml of EPI was applied to a 0.5 ml heparin column. Flow through contained 77% and B wash contained 5 16% of the applied EPI activity. No EPI was detected in eluates with 0.25, 0.75 and 1.5 M NaCl respectively.

(Asp¹ - Thr¹⁶¹)-EPI has one potential N-linked glycosylation site (Asn¹¹⁷) and the importance of this glycosylation for activity was investigated.

10 (Asp¹ - Thr¹⁶¹)-EPI was purified from COS culture medium by affinity chromatography of FXa-Sepharose. In SDS-PAGE the purified protein appeared as a glycosylated band near 27 kDa and an unglycosylated band near 22 kDa (shown by treatment with endoglycosidase F). The glycosylated and unglycosylated forms 15 were separated in unreduced SDS-PAGE and were extracted from the gel. Both forms were active in the EPI assay and showed the same specific activity as judged from the staining intensities in the SDS-gel. Glycosylation at Asn¹¹⁷ is therefore apparently not essential for EPI activity and active (Asp¹ - Thr¹⁶¹)-EPI 20 can thus be obtained in efficient expression systems where mammalian N-linked glycosylation is not obtained, e.g. in prokaryotes, or as unsecreted protein in yeast.

CLAIMS

1. EPI analogue, characterized in that it has a low heparin binding capacity or does not bind to heparin under physiological conditions.
- 5 2. EPI analogue according to claim 1, wherein the heparin binding domain has been deleted or made non-functional by deleting one or more of the amino acid residues in said domain or substituting one or more of the amino acid residues in said domain with other naturally occurring amino acid residues.
- 10 3. EPI analogue according to claim 1 or 2, characterized in that it at least contains the first and second Kunitz domain of native EPI.
4. EPI analogue according to claim 3, characterized in that it at least contains the amino acid sequence from Phe25 to 15 Glu148 of native EPI.
5. EPI analogue according to one or more of the previous claims, wherein one or more of the amino acid residues in the sequence from Asp149 to the C-terminal Met276 of native EPI have been deleted.
- 20 6. EPI analogue according to claim 5 wherein one or more of the amino acid residues in the sequence from Arg246 to Lys265 of native EPI have been deleted.
7. EPI analogue according to claim 1 comprising the amino acid sequence from Asp1 to Arg249 of native EPI.
- 25 8. EPI analogue according to claim 1 comprising the amino acid sequence from Asp1 to Glu235 of native EPI.
9. EPI analogue according to claim 1 comprising the amino acid sequence from Asp1 to Thr161 of native EPI.

10. EPI analogue according to claim 1 comprising the amino acid sequence from Glu15 to Arg249 of native EPI.

11. EPI analogue according to claim 1 comprising the amino acid sequence from Glu15 to Gln235 of native EPI.

5 12. EPI analogue according to claim 1 comprising the amino acid sequence from Glu15 to Thr161 of native EPI.

13. EPI analogue according to any of the previous claims and further having a Ser as the N-terminal amino acid residue.

14. DNA-sequence encoding an EPI analogue according to any of 10 the previous claims.

15. Expression vector containing a DNA-sequence according to claim 14.

16. Transformed or transfected microorganism or cell line comprising a vector according to claim 15.

15 17. Method for preparation of EPI analogues according to any of the previous claims wherein a microorganism or cell line according to claim 16 is cultured in a suitable culture medium whereupon the EPI analogue is isolated.

18. A therapeutic preparation for the treatment of patients 20 having coagulation disorders or cancer, characterized in that it contains an EPI-analogue according to any of the previous claims 1-13 and suitable adjuvants and additions.

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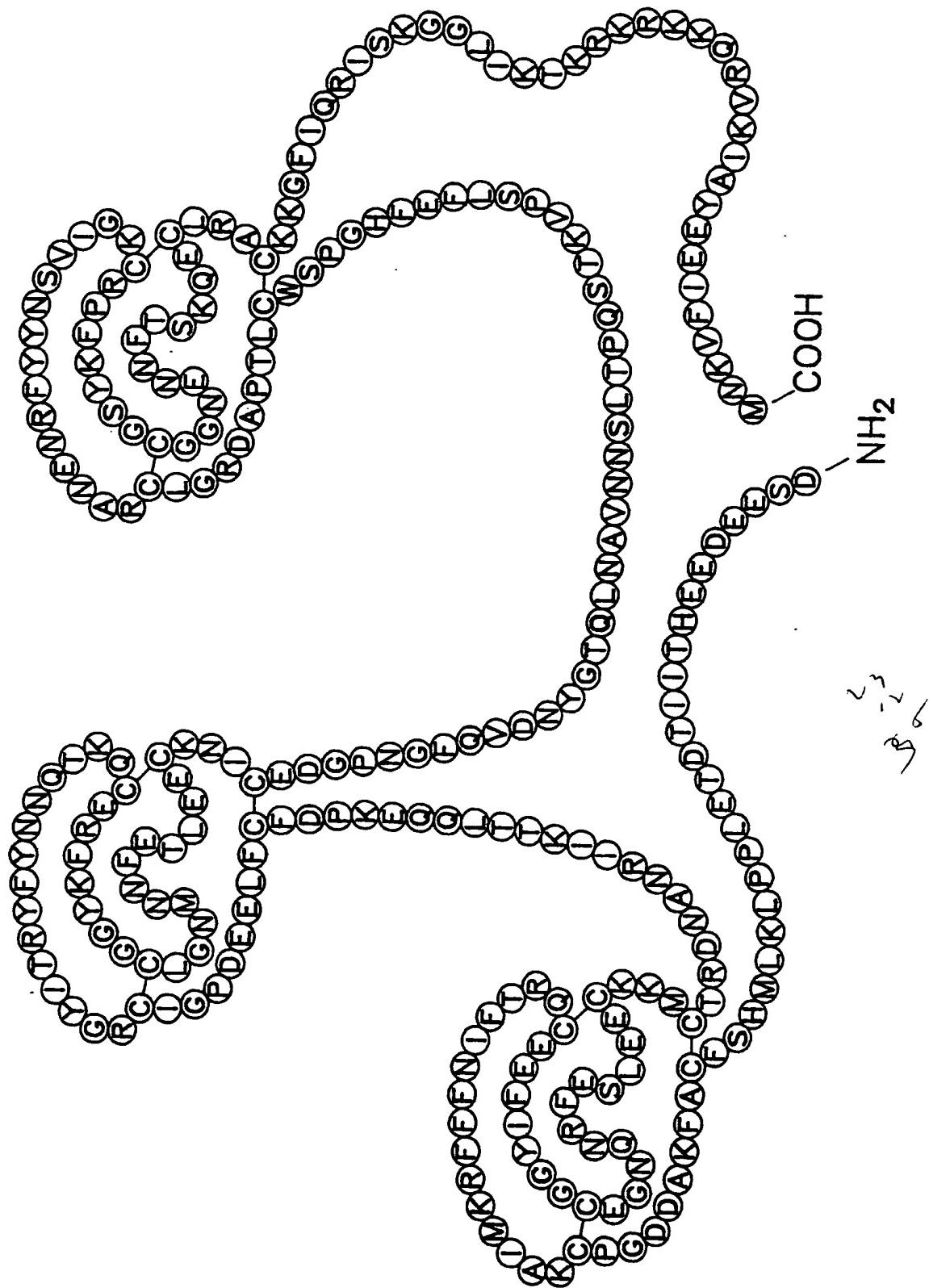


Fig. 1

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Asp	Ser	Glu	Glu	Asp	Glu	Glu	His	Thr	Ile	Ile	Thr	Asp	Thr	Glu	Ile
1				5					10					15	
Pro	Pro	Leu	Lys	Leu	Met	His	Ser	Phe	Cys	Ala	Phe	Lys	Ala	Asp	Asp
				20				25				30			
Gly	Pro	Cys	Lys	Ala	Ile	Met	Lys	Arg	Phe	Phe	Asn	Ile	Phe	Tyr	
				35			40				45				
Arg	Gln	Cys	Glu	Glu	Phe	Ile	Tyr	Gly	Gly	Cys	Glu	Gly	Asn	Gln	Asn
				50		55				60					
Arg	Phe	Glu	Ser	Leu	Glu	Glu	Cys	Lys	Lys	Met	Cys	Thr	Arg	Asp	Asn
	65			70			75				80				
Ala	Asn	Arg	Ile	Ile	Lys	Thr	Thr	Leu	Gln	Gln	Glu	Lys	Pro	Asp	Phe
				85				90				95			
Cys	Phe	Leu	Glu	Glu	Asp	Pro	Gly	Ile	Cys	Arg	Gly	Tyr	Ile	Thr	Arg
				100				105			110				
Tyr	Phe	Tyr	Asn	Asn	Gln	Thr	Lys	Gln	Cys	Glu	Arg	Phe	Lys	Tyr	Gly
				115			120				125				
Gly	Cys	Leu	Gly	Asn	Met	Asn	Asn	Phe	Glu	Thr	Leu	Glu	Glu	Cys	Lys
				130			135				140				
Asn	Ile	Cys	Glu	Asp	Gly	Pro	Asn	Gly	Phe	Gln	Val	Asp	Asn	Tyr	Gly
	145				150			155			160				
Thr	Gln	Leu	Asn	Ala	Val	Asn	Asn	Ser	Leu	Thr	Pro	Gln	Ser	Thr	Lys
				165				170			175				
Val	Pro	Ser	Leu	Phe	Glu	Phe	His	Gly	Pro	Ser	Trp	Cys	Leu	Thr	Pro
				180			185				190				
Ala	Asp	Arg	Gly	Leu	Cys	Arg	Ala	Asn	Glu	Asn	Arg	Phe	Tyr	Tyr	Asn
				195			200				205				
Ser	Val	Ile	Gly	Lys	Cys	Arg	Pro	Phe	Lys	Tyr	Ser	Gly	Cys	Gly	Gly
				210			215			220					
Asn	Glu	Asn	Asn	Phe	Thr	Ser	Lys	Gln	Glu	Cys	Leu	Arg	Ala	Cys	Lys
	225				230			235			240				
Lys	Gly	Phe	Ile	Gln	Arg	Ile	Ser	Lys	Gly	Gly	Leu	Ile	Lys	Thr	Lys
				245				250			255				
Arg	Lys	Arg	Lys	Lys	Gln	Arg	Val	Lys	Ile	Ala	Tyr	Glu	Ile	Phe	
				260			265			270					
Val	Lys	Asn	Met												
			275												

Fig. 2

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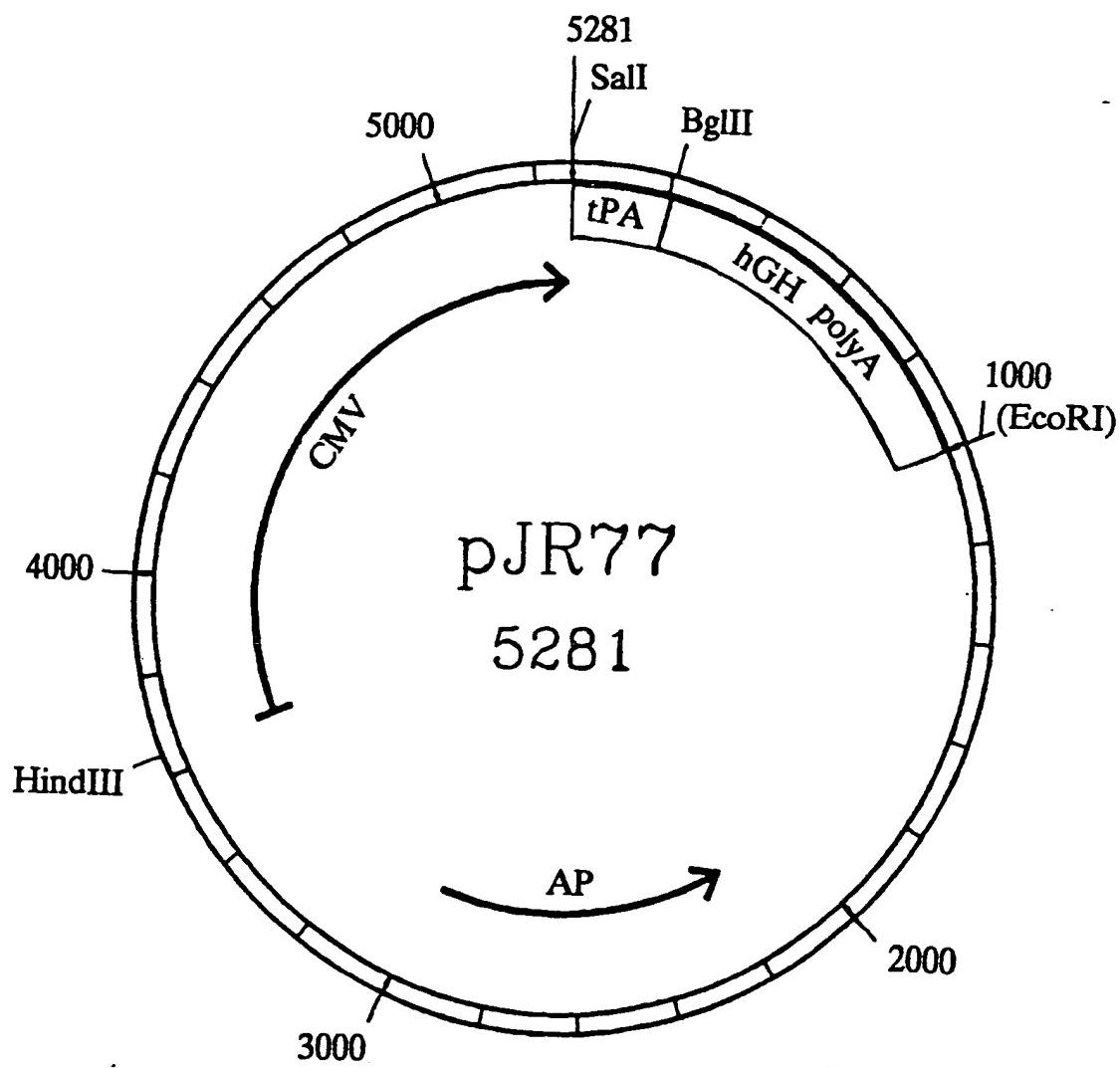


Fig. 3

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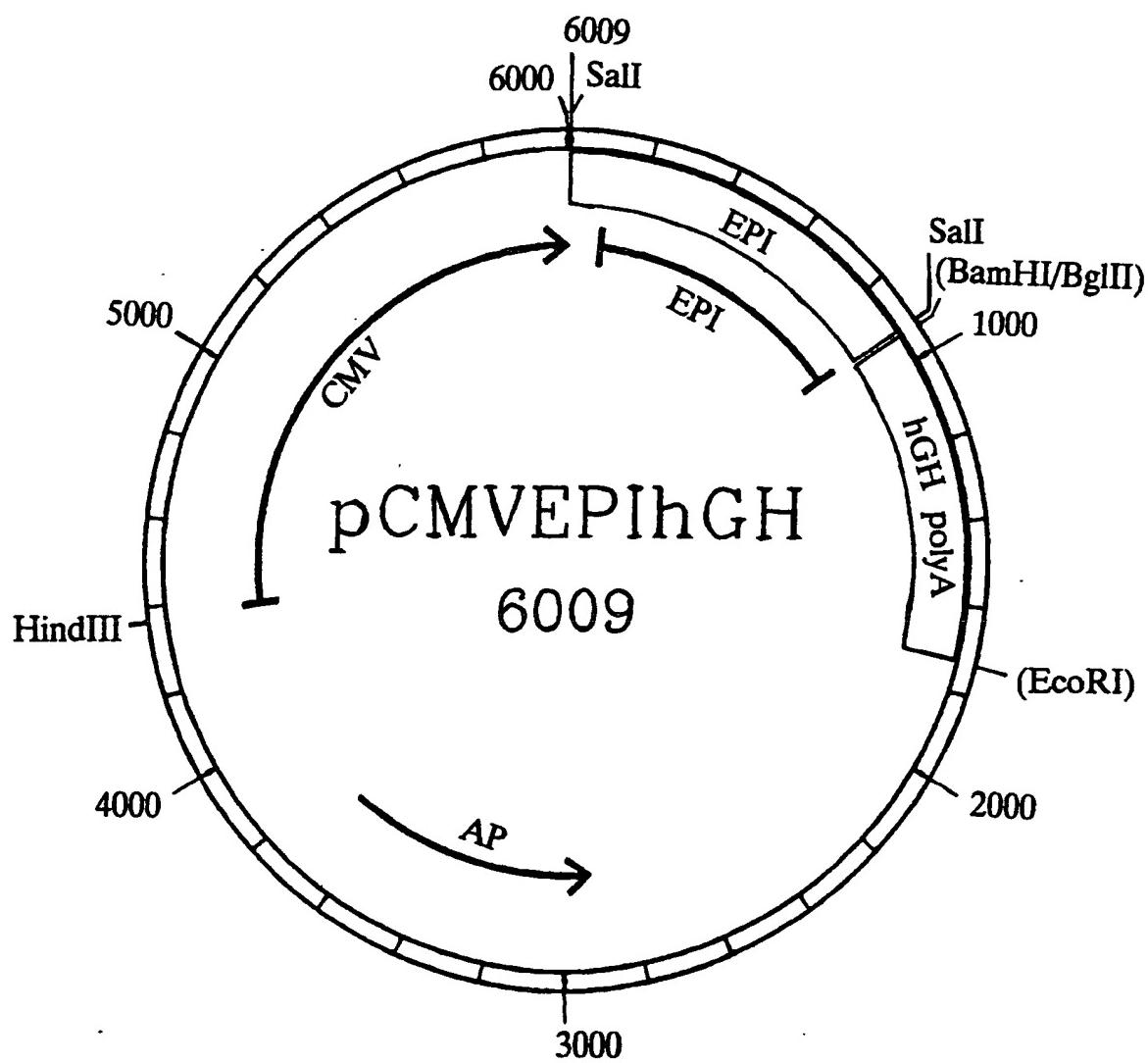


Fig. 4

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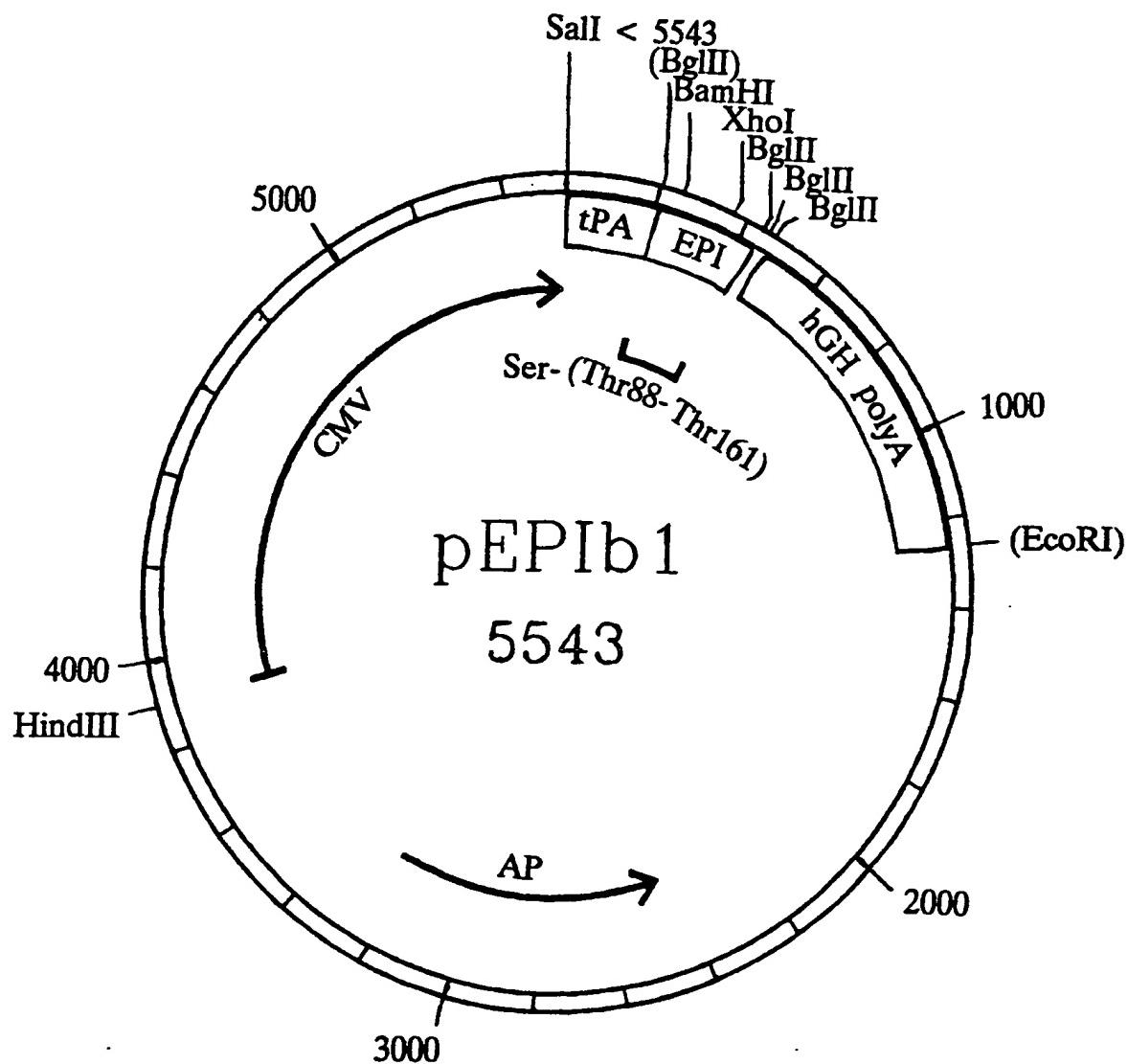


Fig. 5

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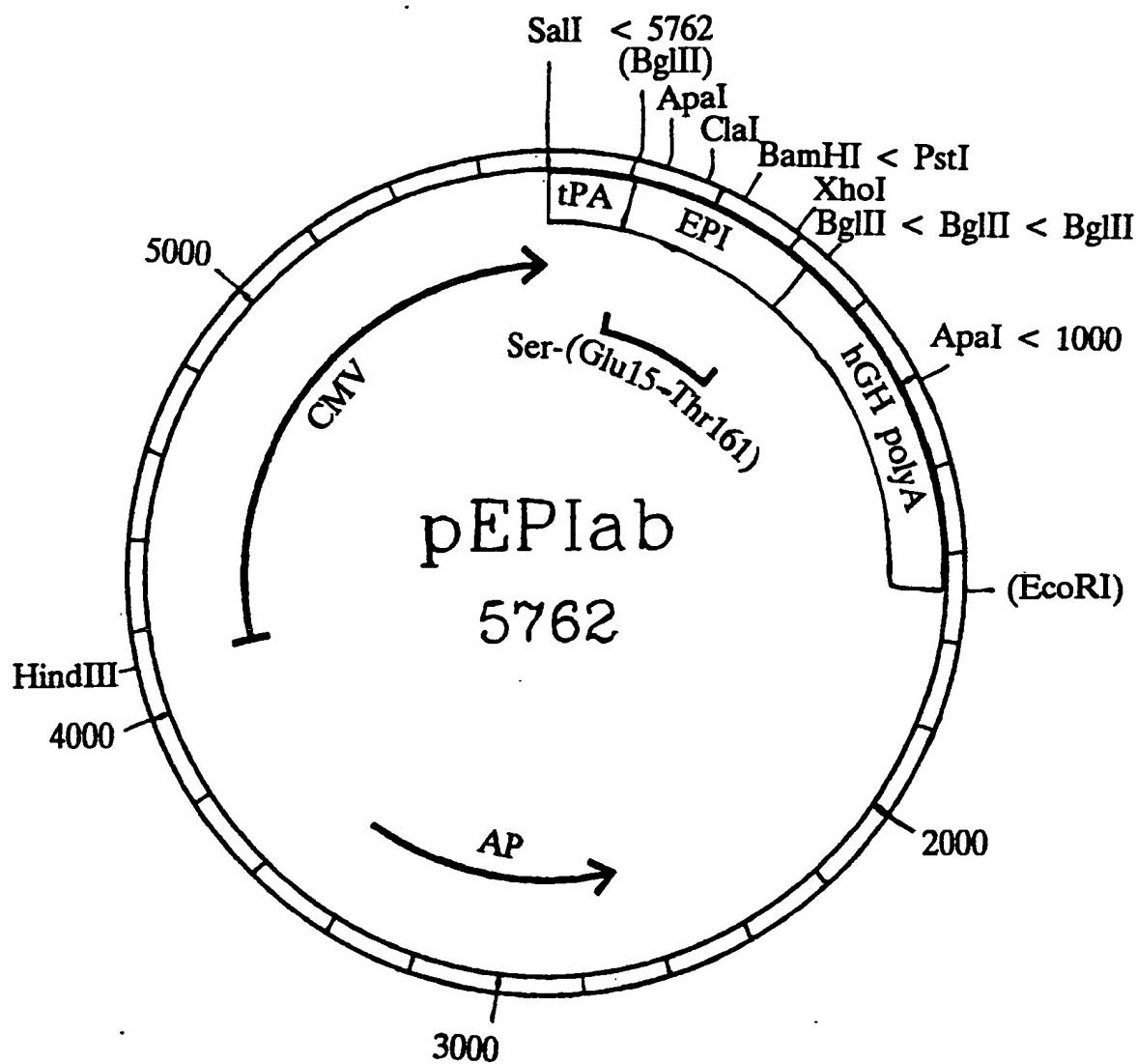


Fig. 6

REPLACEMENT SHEET

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GTCGACAGAG	CTGAGATCCT	ACAGGAGTCC	AGGGCTGGAG	AGAAAACCTC	50
TGCGAGGAAA	GGGAAGGAGC	AAGCCGTGAA	TTTAAGGGAC	GCTGTGAAGC	100
AATC ATG GAT GCA ATG AAG AGA GGG CTC TGC TGT GTG CTG CTG	Met Asp Ala Met Lys Arg Gly Leu Cys Cys Val Leu Leu				143
1	5		10		
CTG TGT GGA GCA GTC TTC GTT TCG CCC AGC CAG GAA ATC CAT	Leu Cys Gly Ala Val Phe Val Ser Pro Ser Gln Glu Ile His				185
15	20		25		
PstI					
GCC CGA TTC AGA AGA GGA GCC AGA TCA ACA CTG CAG CAA GAA	Ala Arg Phe Arg Arg Gly Ala Arg Ser Thr Leu Gln Gln Glu				227
30	35		40		
BamHI					
AAG CCA GAT TTC TGC TTT TTG GAA GAG GAT CCT GGA ATA TGT	Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro Gly Ile Cys				269
45	50		55		
CGA GGT TAT ATT ACC AGG TAT TTT TAT AAC AAT CAG ACA AAA	Arg Gly Tyr Ile Thr Arg Tyr Phe Tyr Asn Asn Gln Thr Lys				311
60	65				
CAG TGT GAA AGG TTC AAG TAT GGT GGA TGC CTG GGC AAT ATG	Gln Cys Glu Arg Phe Lys Tyr Gly Gly Cys Leu Gly Asn Met				353
70	75		80		
XhoI					
AAC AAT TTT GAG ACA CTC GAG GAA TGC AAG AAC ATT TGT GAA	Asn Asn Phe Glu Thr Leu Glu Glu Cys Lys Asn Ile Cys Glu				395
85	90		95		
KpnI					
GAT GGT CCG AAT GGT TTC CAG GTG GAT AAT TAT GGT ACC TGA	Asp Gly Pro Asn Gly Phe Gln Val Asp Asn Tyr Gly Thr End				437
100	105		110		
BglII	BglII	BglII			
AGATCTGAAT	TCTGAAGATC	TAGGCCTATG	AAGATCT		474

Fig. 7

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SalI					
GTCGACAGAG	CTGAGATCCT	ACAGGAGTCC	AGGGCTGGAG	AGAAAACCTC	50
TGCGAGGAAA	GGGAAGGAGC	AAGCCGTGAA	TTTAAGGGAC	GCTGTGAAGC	100
AATC ATG GAT GCA ATG AAG AGA GGG CTC TGC TGT GTG CTG CTG					143
Met Asp Ala Met Lys Arg Gly Leu Cys Cys Val Leu Leu					
1	5	10			
CTG TGT GGA GCA GTC TTC GTT TCG CCC AGC CAG GAA ATC CAT					185
Leu Cys Gly Ala Val Phe Val Ser Pro Ser Gln Glu Ile His					
15	20	25			
SacI					
GCC CGA TTC AGA AGA GGA GCC AGA TCA GAG CTC CCA CCA CTG					227
Ala Arg Phe Arg Arg Gly Ala Arg Ser Glu Leu Pro Pro Leu					
30	35	40			
ApaI					
AAA CTT ATG CAT TCA TTT TGT GCA TTC AAG GCG GAT GAT GGG					269
Lys Leu Met His Ser Phe Cys Ala Phe Lys Ala Asp Asp Gly					
45	50	55			
CCC TGT AAA GCA ATC ATG AAA AGA TTT TTC TTC AAT ATT TTC					311
Pro Cys Lys Ala Ile Met Lys Arg Phe Phe Phe Asn Ile Phe					
60	65				
ACT CGA CAG TGC GAA GAA TTT ATA TAT GGG GGA TGT GAA GGA					353
Thr Arg Gln Cys Glu Glu Phe Ile Tyr Gly Gly Cys Glu Gly					
70	75	80			
ClaI					
AAT CAG AAT CGA TTT GAA AGT CTG GAA GAG TGC AAA AAA ATG					395
Asn Gln Asn Arg Phe Glu Ser Leu Glu Cys Lys Lys Met					
85	90	95			
PstI					
TGT ACA AGA GAT AAT GCA AAC AGG ATT ATA AAG ACA ACA CTG					437
Cys Thr Arg Asp Asn Ala Asn Arg Ile Ile Lys Thr Thr Leu					
100	105	110			
BamHI					
CAG CAA GAA AAG CCA GAT TTC TGC TTT TTG GAA GAG GAT CCT					479
Gln Gln Glu Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro					
115	120	125			
GGA ATA TGT CGA GGT TAT ATT ACC AGG TAT TTT TAT AAC AAT					521
Gly Ile Cys Arg Gly Tyr Ile Thr Arg Tyr Phe Tyr Asn Asn					
130	135				

Fig. 8

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CAG ACA AAA CAG TGT GAA AGG TTC AAG TAT GGT GGA TGC CTG Gln Thr Lys Gln Cys Glu Arg Phe Lys Tyr Gly Gly Cys Leu 140 145 150	563
XhoI	
GGC AAT ATG AAC AAT TTT GAG ACA CTC GAG GAA TGC AAG AAC Gly Asn Met Asn Asn Phe Glu Thr Leu Glu Glu Cys Lys Asn 155 160 165	605
ATT TGT GAA GAT GGT CCG AAT GGT TTC CAG GTG GAT AAT TAT Ile Cys Glu Asp Gly Pro Asn Gly Phe Gln Val Asp Asn Tyr 170 175 180	647
KpnI BglII BglIII BglIII GGT ACC TGA AGATCTGAAT TCTGAAGATC TAGGCCTATG AAGATCT Gly Thr End	693

Fig. 8 cont.

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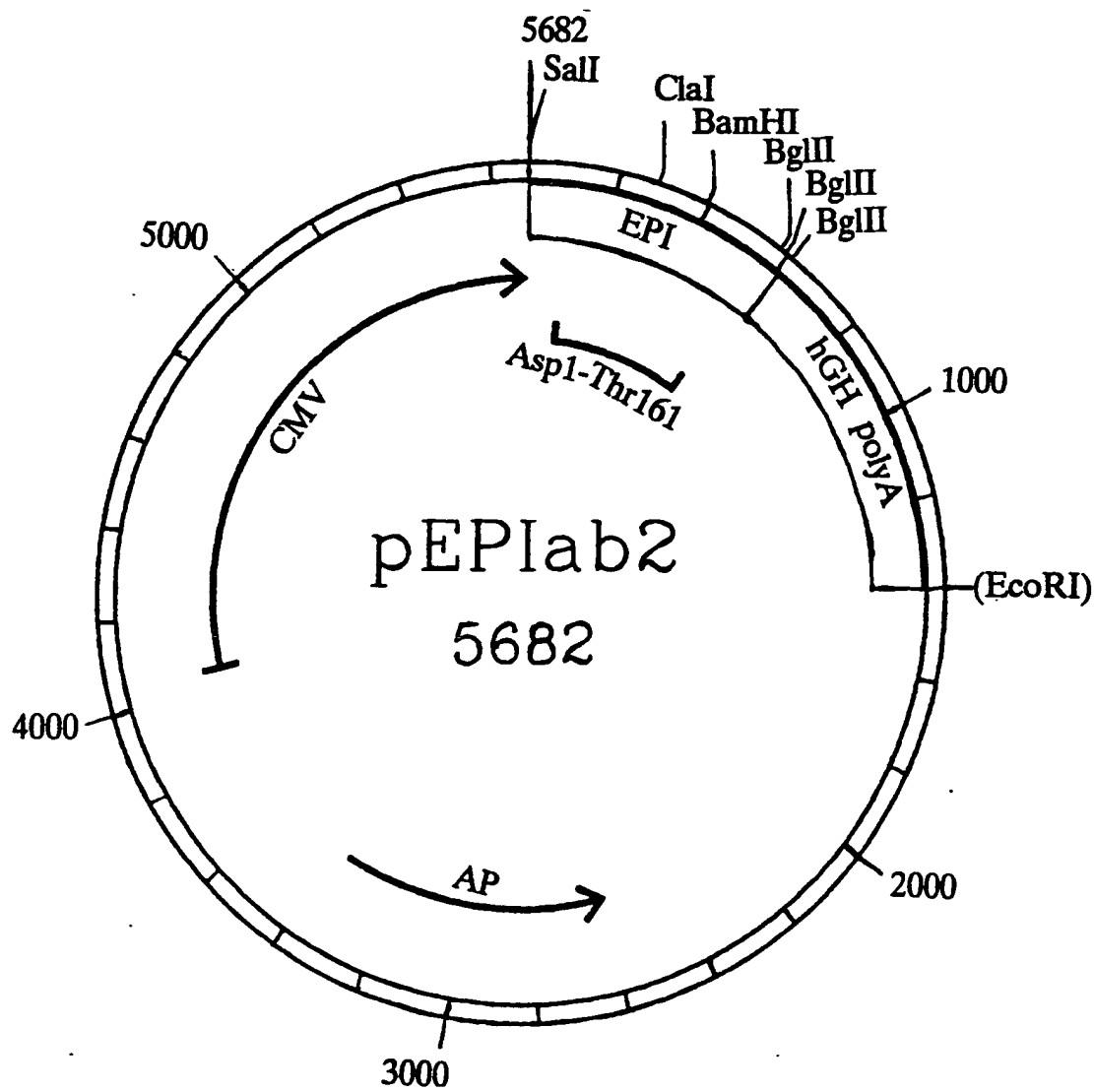


Fig. 9

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SalI

GTCGACC ATG ATT TAC ACA ATG AAG AAA GTA CAT GCA CTT TGG Met Ile Tyr Thr Met Lys Lys Val His Ala Leu Trp	43
1 5 10	

GCT AGC GTA TGC CTG CTG CTT AAT CTT GCC CCT GCC CCT CTT Ala Ser Val Cys Leu Leu Leu Asn Leu Ala Pro Ala Pro Leu	85
15 20 25	

AAT CGT GAT TCT GAG GAA GAT GAA GAA CAC ACA ATT ATC ACA Asn Ala Asp Ser Glu Glu Asp Glu Glu His Thr Ile Ile Thr	127
30 35 40	

GAT ACG GAG CTC CCA CCA CTG AAA CTT ATG CAT TCA TTT TGT Asp Thr Glu Leu Pro Pro Leu Lys Leu Met His Ser Phe Cys	169
45 50	

GCA TTC AAG GCG GAT GAT GGG CCC TGT AAA GCA ATC ATG AAA Ala Phe Lys Ala Asp Asp Gly Pro Cys Lys Ala Ile Met Lys	211
55 60 65	

AGA TTT TTC TTC AAT ATT TTC ACT CGA CAG TGC GAA GAA TTT Arg Phe Phe Phe Asn Ile Phe Thr Arg Gln Cys Glu Glu Phe	253
70 75 80	

ClaI

ATA TAT GGG GGA TGT GAA GGA AAT CAG AAT CGA TTT GAA AGT Ile Tyr Gly Gly Cys Glu Gly Asn Gln Asn Arg Phe Glu Ser	295
85 90 95	

CTG GAA GAG TGC AAA AAA ATG TGT ACA AGA GAT AAT GCA AAC Leu Glu Glu Cys Lys Lys Met Cys Thr Arg Asp Asn Ala Asn	337
100 105	

AGG ATT ATA AAG ACA ACA CTG CAG CAA GAA AAG CCA GAT TTC Arg Ile Ile Lys Thr Thr Leu Gln Gln Glu Lys Pro Asp Phe	379
110 115 120	

BamHI

TGC TTT TTG GAA GAG GAT CCT GGA ATA TGT CGA GGT TAT ATT Cys Phe Leu Glu Glu Asp Pro Gly Ile Cys Arg Gly Tyr Ile	421
125 130 135	

ACC AGG TAT TTT TAT AAC AAT CAG ACA AAA CAG TGT GAA AGG Thr Arg Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg	463
140 145 150	

TTC AAG TAT GGT GGA TGC CTG GGC AAT ATG AAC AAT TTT GAG Phe Lys Tyr Gly Gly Cys Leu Gly Asn Met Asn Asn Phe Glu	505
155 160 165	

Fig. 10

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AGA CTC GAG GAA TGC AAG AAC ATT TGT GAA GAT GGT CCG AAT
 Thr Leu Glu Glu Cys Lys Asn Ile Cys Glu Asp Gly Pro Asn
 170 175

BglII BglII
TCTGAAGATC TAGGCCTATG AAGATCT 614

Fig. 10 cont.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/DK 90/00212

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC
IPC5: C 07 K 13/00, C 12 N 15/15, A 61 K 37/64

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System	Classification Symbols
IPC5	C 07 K; C 12 N; A 61K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in Fields Searched⁸

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	Nature, Vol. 338, 1989 J. Girard et al.: "Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor", see especially p. 518 col. 2 lines 11-12, p. 520 col 1 lines 1-8	1-3,14-16
Y	EP, A2, 0318451 (MONSANTO COMPANY) 31 May 1989, see the whole document	17
Y	---	---
A	EP, A2, 0300988 (WASHINGTON UNIVERSITY) 25 January 1989, see the whole document	1-18
A	---	---

* Special categories of cited documents:¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report

12th November 1990

1990 -11- 19

International Searching Authority

Signature of Authorized Officer

SWEDISH PATENT OFFICE

Mikael G:son Bergstrand

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Thrombosis research, Vol. 48, 1987 B.J. Warn-Cramer et al.: "Partial Purification and characterization of extrinsic ", see page 11 - page 22 ---	1-18
A	Proc. Natl. Acad. Sci., Vol. 84, 1987 George J. Broze, Jr. et al.: "Isolation of the tissue factor inhibitor produced by HepG2 hepatoma cells ", see page 1886 - page 1890 -----	1-18

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers....., because they relate to subject matter not required to be searched by this Authority, namely:

2. Claim numbers.....¹, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The claim states that the EPI analogue has a low heparin binding capacity, without describing anything about the structure of the analogue and hence, the claim is considered not to be clear and concise, c.f. PCT article 6.

3. Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(e).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims. It is covered by claim numbers:
4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- The additional search fees were accompanied by applicant's protest.
 No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 90/00212**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on **90-09-27**
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0318451	89-05-31	AU-D- JP-A-	1928788 1165383
EP-A2- 0300988	89-01-25	AU-D- JP-A-	1928688 1047799